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CHARGE-TRANSFER AND WATER-MEDIATED CHROMATOGRAPHY

I. ELECTRON-ACCEPTOR LIGANDS ON CROSS-LINKED DEXTRAN

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SUMMARY

The adsorption properties of Sephadex gels coupled with different electron-acceptor ligands have been compared with one another and with the unsubstituted matrix gel. The introduction of an acceptor ligand increases the adsorption strength for presumptive donor solutes to an extent that depends on the electron affinity of the immobilized acceptor. We therefore interpret the phenomena observed as being caused primarily by electron transfer, but probably reinforced by other energy-exchange mechanisms such as ion–dipole and dipole–dipole interactions, dispersion forces, hydrogen bonding and water-mediated adsorption, including hydrophobic interactions.

Charge-transfer (CT) chromatography on highly cross-linked dextran derivatives can be used for desalting, "desugaring" and group separations and for the effective separation of aromatic compounds of small molecular size, etc. Charge-transfer chromatography is a separation method that is performed under mild conditions and is thus suitable for the fractionation of substances where the use of gas chromatography, for example, is not feasible. CT chromatography is linear and the capacity can be high. Coloured adsorption complexes have been observed in some instances.

The behaviour of amino acids, peptides, vitamins and a few other substances studied on the CT adsorbents indicates the potentialities of CT chromatography, which can be developed to suit various needs.

However, the adsorption of aromatic and heterocyclic compounds on uncharged hydrophilic gels coupled with electron-acceptor ligands is not likely to be caused by electron charge transfer alone. The water close to the matrix, which presumably is less mobile than the bulk water, may participate in hydrogen bonding, dipole–dipole or ion–dipole interactions with the solutes under investigation and thus be responsible for what may be called *water-mediated adsorption*.

INTRODUCTION

Charge-transfer (CT) chromatography in organic media, which has been dis-

cussed elsewhere[1], has so far not proved promising. In this paper we present the first comprehensive study of CT chromatography on hydrophilic or amphiphilic gels in aqueous media. After the discovery of the molecular-sieving properties of cross-linked dextran (Sephadex)[2], some earlier investigations[3,4] indicated the existence of solute–gel interactions. The phenomena were later studied in considerable detail by Porath and co-workers[5–8], who suggested that the adsorption of the aromatic amino acids might be due to interaction between the π-electrons of the aromatic ring systems and hydrogen or hydroxyl groups in the matrix or in the gel-bound water. Other observed phenomena may be due to charge-transfer effects such as the retention of halogen compounds (*e.g.*, chloroform).

These adsorption phenomena, which do not appear to be related to ionic adsorption, should, if reinforced, be very useful as a new principle of separation. With this aim in mind, some studies were made with dinitrophenyl derivatives of Sephadex. These and other orienting experiments have recently been reported in a short communication[9] and in some reviews[10–12].

Particularly penetrating theoretical and experimental studies have been carried out for benzene–iodine interactions. Some other types of complexes, such as those involving aromatics and aromatic nitro compounds, are more interesting in view of their possible chromatographic applications.

We shall not delve deeply into the theory of molecular complexes, but shall refer to Mulliken and Person's work[13] and to some of the monographs and reviews that have been published in increasing numbers[14–22]. For our purpose it is sufficient to give a brief account of the basic ideas.

Nomenclature

Adsorption of the type treated here is based on the formation of a molecular complex. "Molecular complex chromatography" would therefore be a logically justified term, but it is clumsy, imprecise and too general as it would include, for example, all kinds of bioaffinity chromatography.

When the ligand is a strong electron acceptor or electron donor, it would be appropriate to use the terms "charge-transfer adsorption" or "*c*harge-*t*ransfer *c*hromatography" (abbreviated to CTC*). An alternative terminology would be "electron affinity chromatography", but this is unsuitable and even misleading. The important point is, however, that the selected ligand-gel adsorbent exhibits maximal charge-transfer effects, although we are well aware that factors other than charge transfer may play a significant and sometimes even a dominant role in the adsorption process.

Abbreviations. CT = charge transfer; EDA = electron donor–acceptor; CTC = charge-transfer chromatography; EDAC = electron donor–acceptor chromatography; HOMO = highest occupied molecular orbital; LEMO = lowest empty molecular orbital; EP-Sephadex = epoxypropyl-Sephadex; ANA-Sephadex = aminonaphthalamide-S-Sephadex; PCP-Sephadex = pentachlorophenyl-S-Sephadex;

* *E*lectron *d*onor–*a*cceptor *c*hromatography (EDAC) is also an acceptable alternative and preferable to those who use the concept charge-transfer in a narrow sense where resonance energy makes a major contribution to the stabilization of the adsorption complex.

MHP-Sephadex = γ-mercapto-β-hydroxypropyl-Sephadex; DNP-Sephadex = 2,4-dinitrophenyl-S-Sephadex; DCQ-Sephadex = 4,5-dicyano-2-chloro-*p*-benzoquinone-(1)-S-Sephadex; EDTA = ethylenediaminetetraacetic acid; DMSO = dimethyl sulphoxide.

Theoretical considerations

Molecular compounds or intermolecular complexes are formed as a result of coulombic and polarization forces. Mulliken and Person[13] have shown that in addition to these forces an electron displacement or transfer from one of the components (the donor) to the other (the acceptor) may contribute to the stabilization of the complex.

Two substances, D (donor) and A (acceptor), form the molecular complex AD:

$$A + D \rightleftarrows AD \qquad (1a)$$

The strength of the complex AD is determined by the association constant, K. In order to be able to apply this concept in chromatographic experiments, either of the species A or D must be immobilized to an insoluble matrix support. The immobilization should preferably be carried out by covalent coupling of A or D to the matrix as a "ligand". For example, fixation of A to the matrix will give an adsorbent that will remove D from a surrounding solution by formation of the adsorbate complex AD. If the complex constant is not too large, a molecule of D will travel through a bed of immobilized A (an "acceptor-adsorbent") and, during its passage, will be alternately adsorbed and desorbed. Consequently, D will be retarded to an extent that depends on the association constant, K, the concentration of A (degree of substitution), the permeability of the gel to the solute D (*i.e.*, the space available for the complex AD) and the ratio of gel to free liquid.

The K values given in the literature usually lie within the range 0–100 l mole^{-1} and consequently D may travel independently of its concentration in the bed (linear chromatography). However, as most investigations on charge-transfer reactions have been carried out with non-biological systems, the K values in the literature are of dubious value for reactions in aqueous media. Entirely different conditions prevail in aqueous media and, moreover, the immobilization of one of the interacting species will restrict its molecular motility and will thus influence, in an unpredictable way, the magnitude of the complex constant.

According to Szent-Györgyi[15], Pullman and Pullman[22], Kosower[23] and others, charge transfer may play a role in various biological phenomena. Others are sceptical about the significance of charge-transfer interactions between biomolecules. However, adsorption due to charge transfer between biomolecules and a CT ligand that is deliberately selected to facilitate the electron transfer (processes in CTC) can be demonstrated experimentally, as opposed to what can be expected in living systems or *in vitro* between biochemically important interacting molecules.

As indicated earlier, most experiments on charge transfer have so far been performed in organic solvents. Electron transfer from D to A is likely to be facilitated in polar solvents such as water, as the solvation of the components A, D and AD probably enhances the complex formation in a manner analogous to that in which hydrophobic interactions between non-polar substances are promoted in water.

For chromatography in aqueous media and with an immobilized acceptor, eqn. 1a may be modified to

$$\text{Matrix–O–A}_{(H_2O)_n} + \text{D}_{(H_2O)_m} \rightleftarrows \text{Matrix–O[AD]}_{(H_2O)_r} + (m + n - v)H_2O \quad (1b)$$

This equation does not take into account ionization. Already published results[8] indicate that attraction between certain aromatic biomolecules and strong electron acceptor gels probably cannot be explained even with eqn. 1b. We therefore believe that water ionizes the molecular complex, at least in some instances, to form ion-pair adsorbates:

$$\text{Matrix–O[AD]}_{(H_2O)_r} + pH_2O \rightleftarrows \text{Matrix–O–A}^-_{(H_2O)}\ \text{D}^+_{(H_2O)_{r+p-q}} \quad (1c)$$

or

$$\text{Matrix–O[AD]}_{(H_2O)_r} + pH_2O \rightleftarrows \text{Matrix–O–A}^-_{(H_2O)_p}\text{D}^+_{(H_2O)_{r-p}} \quad (1d)$$

Such ionization of charge-transfer complexes has been shown to occur in polar solvents[18]. Ions and ion radicals may be formed. The solvation contributes to the stabilization of the ion pair. The stabilization energy, $-\Delta H_{H_2O}$, is large and can, for ions of radius 5 Å, be as much as 110 kcal per mole of water[19].

In this introductory stage of our methodological study, we have selected mainly amino acids and peptides as model donors, but we also included some vitamins and heterocyclic compounds to demonstrate that CTC has a wide range of applications. The donor properties dominate for the aromatic substances, and consequently we are at present most interested in synthesizing acceptor adsorbents, *viz.*, immobilized electron acceptors. This can be achieved if one finds suitable (1) acceptors, (2) matrices and (3) methods to attach the acceptors covalently to the matrix. These problems are mutually interdependent but we have already devised some general solutions to the last two problems as a result of many years of systematic studies[24,25].

For guidance in the selection of suitable acceptors, we can start from the following facts. In their simplified valence-bond treatment Mulliken and Person[13] described the ground state and exited state of a molecular complex by the composite time-dependent wave functions ψ_N and ψ_r, respectively:

$$\psi_N(\text{D}\cdot\text{A}) = a\psi_0(\text{D, A}) + b\psi_1(\text{D}^+\text{–A}^-) \quad (2a)$$

(No bond; Dative bond)

and

$$\psi_r(\text{D}\cdot\text{A}) = -b^*\psi_0(\text{D, A}) + a^*\psi_1(\text{D}^+\text{–A}^-) \quad (2b)$$

where ψ_0 and ψ_1 are wave functions for the indicated states and the coefficients a, b, b^* and a^* describe the relative contributions from the different structures. To obtain the energy eigenvalues and construct the energy level diagrams (Fig. 1), it is necessary to solve the Schrödinger equation:

$$H\psi = W\psi \quad (3)$$

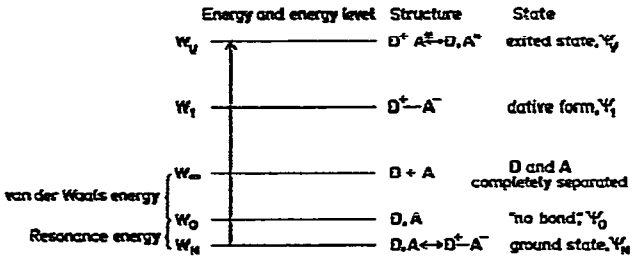


Fig. 1. Energy diagram for the molecular complex D,A. Excitation from the ground level (indicated by the arrow) results in absorption due to both locally excited states and charge-transfer states (ψ_v). The charge-transfer transition band might be in the visible region of the spectrum. Coloured zones have occasionally been observed in CT chromatography. $W_0 - W_N$ is the charge-transfer contribution to the binding energy. $W_\infty - W_0$ is that part of the total binding energy ($W_\infty - W_N$) which depends on dipole–dipole and charge–dipole interactions, dispersion forces, etc.

where ψ, W and H are the eigenfunction, energy eigenvalue and the Hamiltonian operator, respectively. Approximate solutions can be found in special cases.

In the molecular orbital treatment, the "Hückel approximation" for π-electron systems makes it possible to evaluate the energy levels of aromatic, heterocyclic and unsaturated substances. Dewar and Lepley[26] treated weak interactions using perturbation methods. Fig. 2 shows schematically the electron transfer from the highest occupied molecular orbital (HOMO) to the lowest empty (antibonding) molecular orbital (LEMO). To characterize the energy conditions for the formation of a charge-transfer complex, the relative positions of HOMO and LEMO are of decisive importance.

Pullman and Pullman[16] calculated the energy, ε, for a large number of biomolecules according to the equation

$$\varepsilon = \alpha + k_i\beta \tag{4}$$

where α and β are the coulombic and resonance integrals, respectively, and the coefficient k_i is negative for vacant orbitals. With the approximations made by Pullman and Pullman, the resonance integral $\beta \approx 3.26$ in electronvolts, and consequently the resonance energy is 3.26 keV. The term ε_{HOMO} corresponds to the ionization energy of the donor and ε_{LEMO} to the electron affinity of the acceptor. The smaller the energy

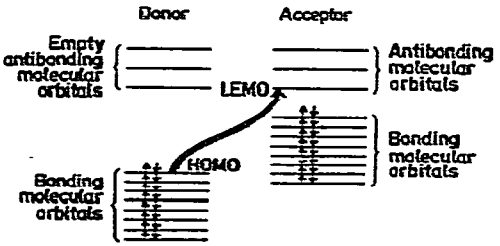


Fig. 2. Energy level diagram for acceptor and donor orbitals. Electron transfer from HOMO of the donor to LEMO of the acceptor and electron spin (indicating occupied orbitals) are marked by arrows. After Dewar and Lepley[26].

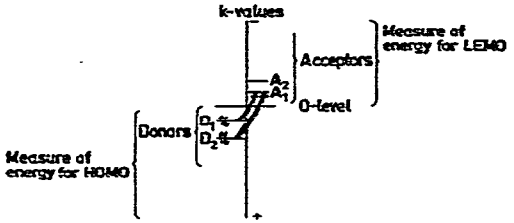
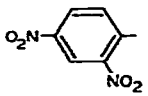


Fig. 3. Principle diagram showing the relative electron acceptor–donor properties (k values according to eqn. 4). The shorter the energy distance between D and A the easier will the electron transfer take place. D_1 gives a stronger complex with A_1 than does D_2.

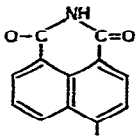
difference, $\Delta\varepsilon$, between HOMO of the donor and LEMO of the acceptor the stronger will be the resonance between the structures D, A and D^+A^-. Fig. 3 shows the principle of the energy level diagrams for HOMO and LEMO for donors and acceptors.

If we confine ourselves to the search for acceptors with π-electrons, we can, with the help of the literature[13–22], differentiate between some especially promising ligand classes:

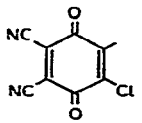
(1) Nitroaromatics, *e.g.*,



(2) Aromatic dicarbonic acid anhydrides and amides, *e.g.*,

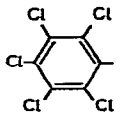


(3) Quinones, *e.g.*,



We have not found any report about the acceptor properties of strongly halogenated aromatic ethers and thioethers, but we nevertheless included the following in our material:

(4) Exhaustively halogenated aromatics, *e.g.*,



The selection of reagents was based on the balance between the acceptor properties, the solubility in a suitable reaction medium and hydrophobicity. The last two properties limit considerably the number of suitable acceptor substances at present. The occurrence of a multinuclear aromatic ring system certainly improves the acceptor properties (k_i in eqn. 4 approaches zero), but at the same time the hydrophobicity increases, resulting in a subsequent decrease in solubility in aqueous media. For these reasons it is necessary to select comparatively simple aromatics or quinones as reagents for the synthesis of hydrophilic acceptor adsorbents.

The water may affect the adsorption process not only as indicated by eqns. 1b and 1c but also by mediation of the presumably hydrogen-bonded, less mobile water close to the matrix. This partly immobilized water appears in some instances to facilitate and in other instances to obstruct the adsorption process.

We can assume that the water-mediated adsorption occurs as follows:

$$\begin{array}{l} MOH \ldots H_2O + S \rightleftharpoons MOH \ldots H_2O \ldots S \rightleftharpoons MOH \ldots H_2O + S \\ \qquad\qquad\qquad\qquad \Updownarrow \\ \qquad\qquad\qquad\qquad MOH + H_2O \ldots S \end{array} \qquad (5a)$$

or

$$M\text{–}X \ldots (H_2O)_m + S(H_2O)_n \rightleftharpoons M\text{–}X \ldots (H_2O)_p \ldots S + (m + n - p)\, H_2O \qquad (5b)$$

where ... indicates weak interactions, S is the solute adsorbed or desorbed, M is the matrix and X is a substituent that will convert the gel into a hydrophobic or amphiphilic adsorbent if it is hydrophobic or will convert the gel into a charge-transfer adsorbent if it is an electron-acceptor donor. In the latter instance eqns. 5b and 1b become identical.

By introducing X, the water may be more tightly bound to the matrix and thereby increase the attraction of adsorbed solute to the hydrated matrix, with an increase in the elution volume as a consequence. The attraction may be caused by hydrogen bonding, dipole–dipole or ion–dipole interactions and depends on the nature of X and S as well as on the composition of the solvent and the temperature.

The water shell around the ligands and matrix is likely to cause steric hindrance to the approach of many solutes closely enough to the ligands for effective electron transfer or other interactions to occur. This may explain the many deviations observed from expected chromatographic behaviour.

EXPERIMENTAL

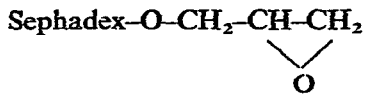
Chemicals

Pentachlorothiophenol was a gift from Bayer (Wuppertal, G.F.R.), and we express our gratitude to Prof. R. Krebs and Drs. Schütz and Plümpe. 1-Chloro-2,4-dinitrobenzene and 2,3-dichloro-5,6-dicyano-*p*-benzoquinone (analytical-reagent grade) were purchased from Merck-Schuchardt (Darmstadt, G.F.R.). 4-Amino-1,8-naphthalamide was obtained from ICN Pharmaceuticals, Life Sciences Group (Plainway, N.Y., U.S.A.).

Bachem Feinchemikalien (Bubendorf, Switzerland) supplied Tyr–Tyr, Tyr_3, Phe_3, Val–Tyr–Val, Glu–Val–Glu, Trp–Trp and His–His. "Hybrin" (Pharmacia, Uppsala, Sweden) was used as a "commercial" sample of ascorbic acid. Chlorpromazine (Hibernal) was a gift from AB Leo (Helsingborg, Sweden), for which we thank Dr. Stig Herbertsson. The amino acid mixture used was the reference standard of Beckman Instruments, (Palo Alto, Calif., U.S.A.). Sephadex G-25 was obtained from Pharmacia. Other chemicals were analytical-reagent grade products obtained from well known chemical companies.

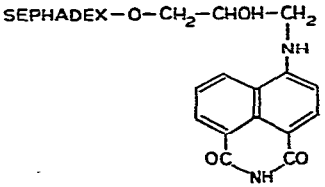
Adsorbents

(a) EP-Sephadex G-25 (epoxypropyl-Sephadex, "activated" Sephadex):



Sephadex (100 g) that was swollen in water and suction-dried was introduced into a three-necked 2-l flask followed by 50 ml of 2 *M* NaOH, 0.2 g of $NaBH_4$ and 5 ml of epichlorohydrin. An additional 50 ml of 2 *M* NaOH and 25 ml of epichlorohydrin were slowly and continuously introduced simultaneously over a period of 4–5 h with moderate stirring. The stirring was continued overnight at room temperature. The gel was collected on a glass filter and washed with water or alkaline solution for immediate use according to the coupling conditions described below.

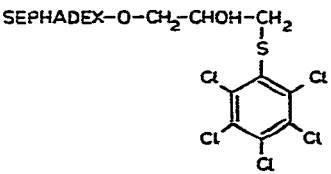
(b) ANA-Sephadex G-25 (aminonaphthalamide-Sephadex):



Wet EP-Sephadex G-25 (100 g) was washed with a mixture of 0.1 *M* NaOH and dimethyl sulphoxide (1:4). The gel was suspended in 200 ml of the NaOH–water–DMSO mixture and 10 g of 4-amino-1,8-naphthalamide were added. The reaction was carried out in a 2-l round-bottomed flask overnight at room temperature with moderate stirring. The gel was washed on a filter successively with DMSO, acetone, ethanol and water.

The gel used in the chromatographic experiments was analysed and found to contain 0.268% *N* or 191 μmole of nitrogen per gram of dry gel substance, *i.e.*, about 95 μmole of coupled ligand. As the analysis of hydrophilic gels is usually difficult (owing to the uncertainty in the residual water after extensive drying and the extreme hydroscopic properties of the dry gel), we estimate the nitrogen content to be about 100 μmole per gram of dry matrix substance.

(c) PCP-Sephadex (pentachlorophenyl-S-Sephadex G-25):



Suction-"dried" and swollen EP-Sephadex G-25 (100 g) was washed with at least 5 volumes of 0.5 *M* NaOH in 50% ethanol containing 0.5 g of $NaBH_4$. The gel was transferred into a 2-l round-bottomed flask equipped with a stirrer. Pentachlorothiophenol (10 g) dissolved in 100 ml of the alkaline solvent was added. The reaction

was allowed to proceed overnight at room temperature. The gel was then washed extensively on a glass filter with warm ethanol, acetone and finally hot water.

The gel used in the chromatographic experiments was found to contain 1.32% S and 6.36% Cl, corresponding to 413 and 1789 μmole per gram, respectively, of dry substance. The molar ratio should be 1:5. The disagreement with the expected ratio is probably due to the uncertainty in the chlorine analysis. The ligand content of approximately 400 μmole per gram of matrix substance is one of the highest among the charge-transfer adsorbents studied up to now.

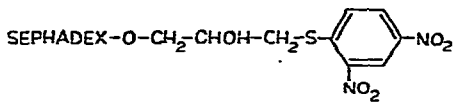
(d) MHP-Sephadex G-25 (γ-mercapto-β-hydroxypropyl-Sephadex G-25):

Sephadex–O–CH_2–CHOH–CH_2–SH

This is an intermediate in the synthesis of DNP- and the quinone gels. The synthesis was carried out as follows.

Swollen EP-Sephadex (100 g) was washed with 0.1 *M* $NaHCO_3$ containing 0.001 *M* EDTA and transferred into a 2-l round-bottomed flask and 50 ml of mercaptoethanol were added. The suspension was stirred for 1 h at room temperature. The gel was washed with 5–6 l of deaerated water and used immediately for coupling with the desired ligand substance.

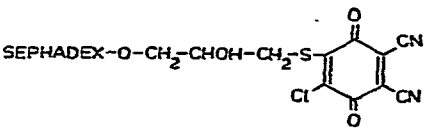
(e) DNP-Sephadex, 2,4-dinitrophenyl-S-Sephadex:



Wet MHP-Sephadex G-25 (100 g) was washed with an ethanolic solution consisting of 0.1 *M* $NaHCO_3$ in 50% ethanol containing 0.001 *M* EDTA. The gel was transferred into a 2-l reaction flask, then 100 ml of the ethanolic solution were added together with 1.5 g of 1-chloro-2,4-dinitrobenzene. The reaction was allowed to proceed for 2 h at room temperature with stirring. The gel was washed extensively with water, ethanol, acetone and water, in that order.

This gel is usually yellow but may occasionally turn green, in which event it should be washed with acidic buffer (pH about 3) in order to obtain a permanent yellow colour. The gel used in our experiments was found to contain 1.35% S and 0.56% N ,corresponding to 420 μmole of sulphur and 400 μmole of nitrogen, respectively, per gram of dry substance. The ligand content was thus about 200 μmole per gram of matrix substance and the yield was *ca.* 45% based on the sulphur.

(f) DCQ-Sephadex (4,5-dicyano-2-chloro-p-benzoquinone-S-Sephadex), with the most likely main substituent:



(i) Method I (high substitution). Freshly prepared MHP-Sephadex (100 g) was extensively washed with 0.1 *M* $NaHCO_3$ and reduced with $NaBH_4$ (0.5 g per 100 ml

of 0.1 *M* $NaHCO_3$) for 15 minutes and then washed with 2–8 volumes of 1.1 *M* $NaHCO_3$. The gel was suspended in 100 ml of 96% ethanol and transferred into a 2-l round-bottomed flask equipped with a stirrer. A 4-g amount of 2,3-dichloro-5,6-dicyano-*p*-benzoquinone was added and the reaction was allowed to proceed at room temperature overnight. The gel was washed with 3 l of water at room temperature and then with 200 ml of hot ethanol, 200 ml of hot water, 100 ml of hot acetone and finally with 1 l of hot water.

The reddish brown gel was found to contain 2.29% S, 0.506% N and 0.73% Cl, corresponding to approximately 200 μmole of quinone substituent and 500 μmole of excess sulphur per gram of dry gel substance.

(ii) Method II (low substitution). The synthesis was the same as in method I except that the intermediate MHP-Sephadex was prepared using only 10 ml of mercaptoethanol instead of 50 ml. The gel was found to contain 0.259% N and 0.22% Cl, corresponding to 184 and 62 μmole of nitrogen and sulphur, respectively, per gram. We estimate the substitution to be about 75 μmole of ligand per gram of dry gel substance.

Methods

Fractionation experiments with artificial amino acid mixtures. These experiments were performed on two columns coupled in series, each column having the dimensions 39.5 × 1.4 cm ($V_T \approx 62$ ml). The beds consisted of pentachlorophenyl-S-Sephadex and dicyanochloro-*p*-benzoquinone-S-Sephadex in 0.01 *M* $NaHCO_3$ (pH 9.0) or 0.1 *M* NaOAc (pH 5.0). The experiments were performed at room temperature. A 5.5-ml volume of the Beckman amino acid reference mixture was evaporated to dryness and the amino acids were dissolved in 5.5 ml of equilibrating buffer to which tryptophan was added to about 110 absorbance units (280 nm).

A 0.5-ml volume of sample was introduced into the column, followed by elution with equilibrating buffer at a rate of 4.2 ml/h until 1.5 V_T had passed, and thereafter the flow-rate was increased to 10.2 ml/h. Fractions of 1.4 and 3.4 ml, respectively, were collected (a Fractomat FRS fraction collector from Stålprodukter, Uppsala, Sweden, was used throughout this study). Aliquots of 0.5 ml were taken for ninhydrin analysis according to Moore and Stein[33]. The absorbances at 230 and 280 nm were also measured. The amino acids in pooled fractions were then determined with a Durrum D-500 Amino Acid Analyzer.

Exploratory adsorption experiments. These experiments were aimed at investigating the characteristics of the gels and were carried out on columns of two different sizes. All experiments in which $V_E/V_T > 10$ and those where 4 *M* NaCl was used were carried out on short columns (6.0 × 1.02 cm); all other experiments were carried out using a Pharmacia K-15 column with a bed of 23.5 × 1.5 cm ($V_T \approx 40$ ml). The eluents used were 0.1 *M* sodium formate (pH 3.2) or 0.1 *M* ammonium carbonate (pH 8.8). Typically, a 0.5–1.0-ml sample containing 10 absorbance units (280 nm) was introduced into the bed. The chromatogram was developed at a flow-rate of 10 ml/h. The effluent was allowed to pass through an LKB Uvicord, Type 4701A. The experiments were performed at room temperature (20 ± 1°) and in a cold-room (4°).

RESULTS

Study of the adsorption properties of common amino acids

Fig. 4 shows the elution profile of the test amino acid mixture after chromatography on the quinone gel. The test sample contains all of the common amino acids usually present in protein hydrolysates. The result shows that the quinone gel resolves the amino acid mixture into distinct groups separated according to the molecular structure with respect to π-electron, lone electron pair content and charges (interfering with the π-electron cloud of the benzene or imidazole ring systems).

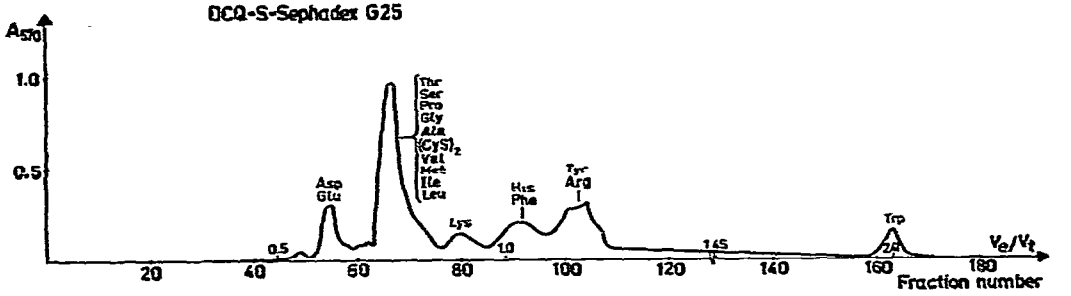


Fig. 4. Fractionation of a synthetic amino acid mixture at pH 5.0 (0.1 *M* sodium acetate), 20°, on DCQ-Sephadex G-25.

Studies of various factors affecting the adsorption

In order to investigate further how the adsorption depends on various chemical and physical factors, a number of simple model substances were run. The experiments were carried out on different gels, at two temperatures (4 and 20°), at two pHs (3.2 and 8.8), and in some instances in the presence of a high concentration of salt (4 *M* NaCl). "Control experiments" were carried out on Sephadex G-25.

Table I summarizes the results of this study. It is compiled from the results obtained with short columns with different acceptor gels of different degrees of substitution. The errors in the V_E/V_T values are calculated to be within about 10%, and we therefore believe that the results can be used for meaningful comparisons and semi-quantitative evaluation of the different adsorption characteristics that we will discuss separately. All substances in virtually all experiments travelled through the beds as well defined zones and were eluted as symmetrical peaks. The adsorption is thus linear, except possibly for substances that are very strongly adsorbed ($V_E/V_T > 30$).

Adsorption to the matrix support. A comparison between Sephadex G-25 and glycerol-substituted Sephadex G-25 showed that treatment of the former with epichlorohydrin followed by alkaline hydrolysis results in a moderate increase in adsorption of indole derivatives and other aromatic substances (results not presented in this paper).

Effect of temperature. With few exceptions, the adsorption increased as the temperature is decreased from 20 to 4°. The most prominent exceptions are Phe, Phe–Phe and Phe_3, which in all instances examined showed the opposite temperature dependence.

Effect of pH. A comparison of the adsorption displayed by the model sub-

TABLE I

EFFECTS OF TEMPERATURE, pH AND SALT CONCENTRATION ON ADSORPTION USING DIFFERENT GELS

Substance	*Adsorbent and ligand concentration (μmole/g)*										
	Sephadex G-25: 0		*PCP-Sephadex G-25: ≈400*					*DNP-Sephadex G-25: ≈200*			
	pH 3.2,	*pH 8.8,*	*pH 3.2*			*pH 8.8*		*pH 3.2*		*pH 8.8*	
	20°	*20°*	*4°*	*20°*	*20°**	*4°*	*20°*	*4°*	*20°*	*4°*	*20°*
Trp	1.7	1.7	3.7	2.8	7.9	2.6	2.6	3.9	2.7	2.8	2.5
Trp–Trp	3.4	2.7	>40	19.0	>40	—	14.0	>30	>30	>30	4.3
Dopamine	1.1	1.3	1.6	1.6	4.0	1.5	1.3	1.6	1.6	1.6	1.6
Tryptamine	1.2	1.8	4.2	3.3	17.0	4.0	3.8	3.8	3.2	5.0	3.3
Serotonin	1.9	2.1	4.9	3.7	14.0	4.4	3.6	5.6	4.1	7.8	5.3
Tyr	1.0	1.0	1.2	1.1	2.3	0.9	1.0	1.1	1.1	1.0	1.0
Tyr–Tyr	1.5	1.2	2.6	2.0	12.0	1.4	1.5	3.4	3.2	1.8	1.7
Tyr–Tyr–Tyr	1.7	1.4	5.9	4.6	35	2.6	2.6	9.4	7.4	4.3	3.7
His	0.8	1.0	0.8	0.7	1.3	0.8	0.8	0.6	0.6	0.6	0.7
Phe	1.0	1.0	1.1	1.0	1.7	0.8	1.2	0.9	0.9	0.7	1.4
Phe–Phe	—	—	—	—	—	1.4	2.0	—	—	1.1	2.0
Phe–Phe–Phe	—	1.1	—	—	—	2.8	3.9	—	—	1.8	2.8
Val–Tyr–Val	0.9	0.8	1.0	1.4	—	0.7	0.8	0.9	1.5	0.6	0.7
Glu–Tyr–Glu	1.0	0.8	1.5	1.4	1.9	0.8	0.6	1.5	1.5	0.8	0.6

* 4 *M* NaCl included in the eluent.

stances showed a weak pH dependence for tyrosine, tryptamine, dopamine and tryptophan. This dependence is more marked for the peptides (Table I). It is interesting that whereas tyrosine, tryptophan and peptides containing these amino acids are adsorbed more firmly at low pH on the unsubstituted "matrix gels", the opposite is true for dopamine, tryptamine and serotonin. Histidine is more strongly adsorbed at higher than at low pH. In general, the adsorption effects are influenced to the same extent both on the "matrix gels" and on the acceptor gels, but the behaviour of some of these substances are at variance with this conclusion; thus, with tryptamine, dopamine and serotonin the adsorption increment has the opposite sign on pentachlorophenyl-S-Sephadex.

Effect of salt. The influence of salts on the CT adsorption has been mentioned earlier[8,12]. It can be large, as seen in Table I. Trityrosine, for example, is so strongly adsorbed at 20° and pH 3.2 in 4 *M* NaCl that the adsorption is no longer linear. The salt dependence is very different for different substances, a fact that can be used to advantage in practical applications, for example, to increase the adsorption capacity and separation power. It thus appears impossible that the salt effect might be used for desalting and exchange of buffer systems. However, assuming $V_E/V_T \gg 1$, the advancing rear salt boundary will travel at a higher speed than the adsorbed donor substance (see below).

Experiment with chlorpromazine

A column of ANA-Sephadex G-25 gel ($V_T \approx 5.0$ ml) was prepared in 0.1 *M*

DCQ-Sephadex G-25: ≈70					*DCQ-Sephadex G-25: ≈200*			
pH 3.2			*pH 8.8*		*pH 3.2*		*pH 5, pH 7,*	
4°	*20°*	*20°**	*4°*	*20°*	*4°*	*20°*	*20°*	*20°*
3.4	3.3	6.4	3.0	1.9	9.3	6.7	3.2	3.0
>30	>30	>30	4.8	—	>30	>30	—	—
3.6	3.2	3.3	3.3	2.5	9.6	9.5	8.6	>30
9.0	7.7	11.0	6.0	5.3	>30	>30	11.0	19
14.0	9.8	12.0	8.7	6.7	>30	>30	12.5	22
1.5	1.4	2.0	1.0	1.1	2.4	2.2	1.7	1.4
—	4.6	5.2	1.0	1.0	—	—	2.3	2.4
14.0	9.1	21.0	1.4	1.4	>30	>30	—	—
—	1.5	1.1	—	0.8	3.4	2.8	1.8	1.5
1.1	1.3	1.3	1.0	0.9	1.4	1.8	1.6	1.5
—	—	—	—	0.9	—	—	—	—
—	—	—	—	1.1	—	—	—	—
1.9	1.8	—	0.6	0.6	4.6	4.6	1.5	6.3
—	1.8	1.9	0.6	0.6	5.2	4.2	1.1	1.0

sodium formate buffer (pH 3.2). About 1000 absorbance units (A_{255}) of chlorpromazine in 50 μl of buffer were introduced into the column. The column was eluted with the formate buffer at a flow-rate of 13.5 ml/h. Fractions of 2.25 ml were collected and the absorbance at 255 nm was measured. As shown in Fig. 5, a main component was separated from four more rapidly moving sub-components or impurities.

Experiment with ascorbic acid

Two pellets of Hybrin were dissolved in 10 ml of 0.1 *M* sodium formate buffer (pH 3.2) and the solution was centrifuged. A 3-ml volume of the clear supernatant was applied to the same ANA-Sephadex G-25 gel as in the previous experiment, but with a bed of $V_T = 42$ ml (tube I.D. = 1.5 cm). The column was eluted with the formate buffer at a flow-rate of 11.2 ml/h and fractions of 2.8 ml were collected. The absorbance at 282 nm was measured and the absorption spectra were determined for the substances in the peak tubes. Fig. 6 shows the chromatogram with the absorption maxima marked. In this instance the impurities travelled more slowly than the main component.

Separation of some heterocyclic compounds

A 1-ml volume of a mixture of alloxane, barbituric acid, caffeine, xanthine and benzimidazole, each with an absorbance in the range of 4–20 units at 265 nm (200 μmole/g), was introduced into a column (26 × 1.5 cm) of DCQ-Sephadex G-25 in 0.1 *M* ammonium formate buffer (pH 3.2) at room temperature. The chromato-

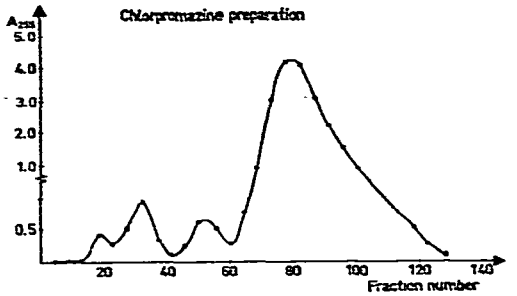


Fig. 5. Chromatogram of a chlorpromazine preparation on ANA-Sephadex in sodium formate buffer, pH 3.2.

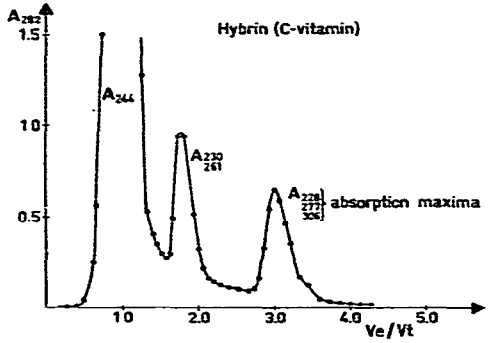


Fig. 6. Chromatogram of an ascorbic acid preparation (Hybrin) on ANA-Sephadex in sodium formate buffer, pH 3.2.

gram was developed at a flow-rate of 11.0 ml/h and 2.75-ml fractions were collected. The absorbance at 265 nm was measured and the elution profile is shown in Fig. 7 (full line). In a similar run with xanthine and imidazole (measured at 240 nm), the distribution shown by the dotted line in Fig. 7 was obtained (see also Table II).

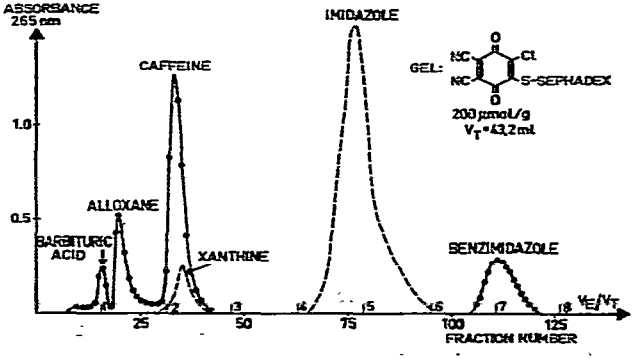


Fig. 7. Separation of an artificial mixture of some heterocyclic compounds on DCQ-Sephadex G-25 at 20° in 0.1 *M* ammonium formate buffer, pH 3.2.

Separation of some B vitamins

An artificial mixture of the vitamins pyridoxine, folic acid, riboflavine and thiamine, each in amounts corresponding to between 10 and 20 absorbance units at 280 nm, and B12 (cyanocobalamin), 4.3 absorbance units at 540 nm, was dissolved in 1.1 ml of 0.1 *M* ammonium acetate (pH 5.0). The sample was applied to a column (2.5 × 1.5 cm) of DCQ-Sephadex G-25 (200 μmole/g) in the buffer and the column was eluted at 20° with 0.1 *M* ammonium acetate. Fractions of 2.7 ml were collected and the absorbance was measured (Fig. 8).

Desalting and "desugaring" experiment

In order to show the possibility that a substance with $V_E/V_T \gg 1$ can be isolated from a solution with a high concentration of salt and neutral substances with $V_E/V_T < 1$, a model experiment was performed with 0.25% serotonin dissolved in 4 *M* NaCl and containing 5% of glucose. A sample of 5 ml was introduced into a column (9.1 × 1.4 cm) of pentachlorophenyl-S-Sephadex G-25 in 4 *M* NaCl and 5% glucose. The sample was followed by 20 ml of 4 *M* NaCl in 0.1 *M* ammonium acetate buffer (pH 5.0) and then by distilled water. The experiment was carried out at room temperature at a flow-rate of 11.4 ml/h and 1.9-ml fractions were collected. The distribution of UV-absorbing material in the fractions was measured at 280 and 250 nm, the glucose by the orcinol method and the salt by conductivity measurements. The absorbance spectra for the peak tubes (6 and 22) were also determined.

Two fractions absorbing at 250 and 280 nm (with different absorbance ratios) were obtained (Fig. 9). The more retarded main component was found to be serotonin, whereas the other was an unidentified impurity. The glucose and NaCl were separated from the serotonin to the extent of more than 99.9%. The tail of the serotonin peak seems to contain impurities with absorbance spectra similar to that of serotonin.

DISCUSSION

In this work we confined ourselves to acceptor adsorbents, but donor adsorbents are also very promising[12]. Obviously the present study is preliminary and incomplete and should therefore be considered only as one of the first steps in a series of systematic attempts to find practical working conditions for electron charge-transfer dependent chromatography. Much more remains to be done before we shall

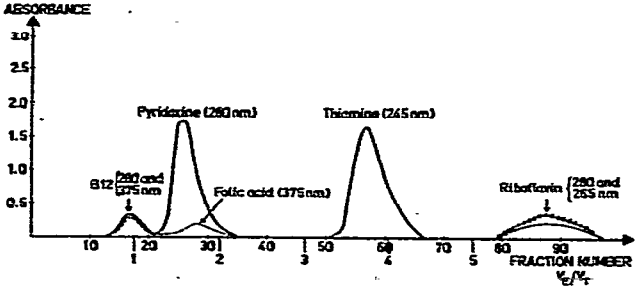


Fig. 8. Chromatogram obtained for an artificial mixture of B vitamins on DCQ-Sephadex. The run was made at 20° in 0.1 *M* ammonium acetate, pH 5.0.

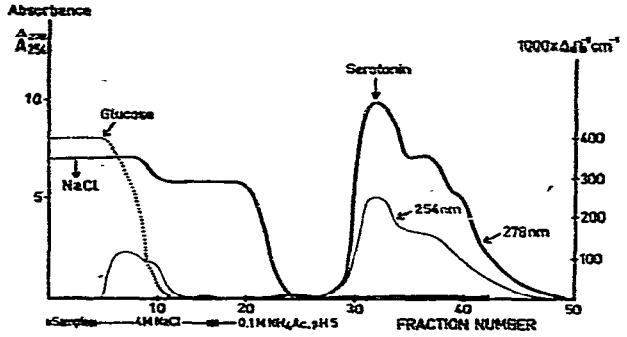


Fig. 9. Desalting and desugaring of serotonin containing 5% glucose and 4 *M* NaCl. The run was made on a column (9.1 × 1.5 cm) of PCP-Sephadex at 20°.

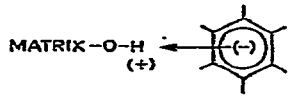
be in a position fully to exploit the great flexibility of CT chromatography. We cannot overemphasize the complexity of the adsorption properties revealed by our data. This complexity is due to the interplay of many simultaneously and cooperatively interacting adsorption-promoting phenomena.

It is obvious that the introduction of acceptor ligands into Sephadex in most instances reinforces the adsorption of π-electron-rich solutes, even if interesting exceptions have been found among our test materials. This fact indicates that similar interactions might be operative in the affinity of solutes for the matrix structure itself. Let us therefore first discuss the adsorption properties of the unsubstituted matrix gels and then compare them with those of the derived adsorbents.

Adsorption to the gel matrix

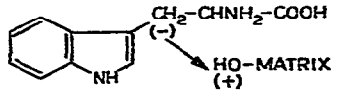
All of the gels described in this paper are based on the extensively cross-linked dextran Sephadex G-25. We have also made preliminary studies with agarose and cellulose, the results of which will be published later. Sephadex contains glucose units connected by glucosidic linkages. In addition, cross-links of the glycerol diether type and glycerol ether substituents (–O–CH_2–CHOH–CH_2OH) are present. Carboxylic and other ionogenic groups are virtually absent. Despite these facts, adsorption phenomena do occur with cross-linked dextran and one must therefore assume that either oxygen, primary or secondary hydroxyl groups or matrix-bound water molecules act as adsorption centres. In the activation with epichlorohydrin used in the synthesis of acceptor adsorbents, one cannot avoid introducing an excess of residual oxirane substituents, which, upon hydrolysis, increase the concentration of glycerol groups in the polysaccharide network. These increase the inherent affinity for π-electron-rich substances such as tryptophan. The reciprocal order in the relative elution volumes of Phe, Tyr and Trp and their k_i values in eqn. 4 (0.91, 0.79 and 0.53, respectively) indicates that Sephadex G-25 functions as a π-electron acceptor. This is due to the presence of hydroxyl groups in the matrix (or to bound water). Support for this hypothesis can be found in the literature, where, for example, the mutual interaction between alcohols and π-electron donors has been described. Tamres[27] has shown a frequency shift in the stretch vibrations of the hydroxyl of methanol when benzene, mesitylene and other aromatics are added as electron donors. Similar ob-

servations for *n*-butanol have been reported by Josien and Sourisseau[28] and Basila *et al.*[29], and one can also cite Mulliken and Person[13]. The structure of an aromatic–hydrophilic adsorption complex can be depicted symbolically as



The distance between the interacting charge centres should be within the range 2–4 Å in all cases of charge transfer.

Tryptophan, with an increased electron charge on C_3 of the indole ring[30], can be represented as



Hydrogen bonding of the type indicated in the previous formula is possible for tyrosine, but the phenolic hydroxyl group may also be hydrogen-bonded to either oxygen or hydroxyl groups in the matrix. The phenolate ion is apparently a weaker donor than the non-ionized phenol, as is evident from the V_E/V_T values of Tyr_2 and Tyr_3.

The adsorption phenomena observed on cross-linked dextran are complex in nature and may involve water that is strongly bound to the gel, in addition to direct interactions with ether oxygen and hydroxyl groups. Consequently, adsorption is mainly accounted for by hydrogen bonding and thus amounts to a special form of proton–electron charge transfer.

Adsorption to the acceptor gels

Upon fixation to the matrix, the ligand substance will undergo a change in its electron affinity, especially if a substituent alteration takes place at or near the π-electron-rich ring system. As close contact is necessary (2–4 Å) for charge transfer to occur between solute and ligand, it seems obvious that steric effects of the matrix might be important. The restricted molecular motility of the ligand and the steric hindrance caused by the matrix might decrease or prevent orbital overlapping and might also change the kinetics of complex formation. The consequences of these factors cannot be predicted. We therefore emphasize our finding that the relative elution volumes (V_E/V_T) of many donor substances seem to correlate with the electron affinities of the ligands when the degree of substitution and the ε_{HOMO} of analogous or similar acceptors are taken into account. The sequence of increasing acceptor potency seems to be as follows: Sephadex < ANA-Sephadex < PCP-Sephadex < DNP-S-Sephadex < DCQ-Sephadex (in acidic and neutral solution). Among the ligands tested, the quinone ligand in DCQ-S-Sephadex has by far the strongest acceptor power as measured at pH 3.2 and 5.8.

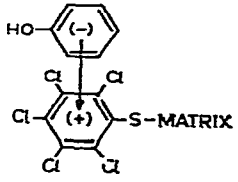
A more rigorous proof that charge-transfer adsorption does in fact occur requires quantum mechanical calculations and also a more comprehensive experimental study in which the contributions due to other adsorption factors have been eliminated or have been quantitatively accounted for.

In a few instances the formation of coloured complexes has been noticed[12]. For example, if serotonin or pyrogallol is introduced into a bed of DNP-S-Sephadex

at high concentration, one observes a red zone against a yellow background. The red zone migrates through the bed and the substance elutes in a colourless solution with retention of its characteristic UV spectrum. In other instances it is not easy to find evidence for the formation of a charge-transfer adsorption complex, but we can often eliminate certain other adsorption phenomena as main causes for the observed effects. However, when ionization takes place according to eqn. 1c, a charge-transfer complex may have only a transient existence.

The ligands used are more or less hydrophobic and hydrophobic adsorption can therefore be expected. The influence of temperature on the adsorption can serve as a guide in estimating the relative importance of hydrophobic interaction *versus* electron charge transfer (and similarly influenced adsorption effects). In the former instance, V_E/V_T would increase when the temperature is increased, whereas the opposite would be expected for charge-transfer adsorption (and hydrogen-bond-dependent adsorption). No comprehensive study of the temperature effects has yet been made, but most experiments, including those documented in Table I, show that adsorption increases when the temperature is decreased. A few exceptions do occur. At pH 8.8, mono-, di- and triphenylalanine are adsorbed more strongly to pentachlorophenyl-S-Sephadex at 20° than they are at 4°. The temperature effects tend to support the hypothesis that electron charge transfer is usually more important than hydrophobic interaction in the adsorption of solutes to the electron-acceptor gels.

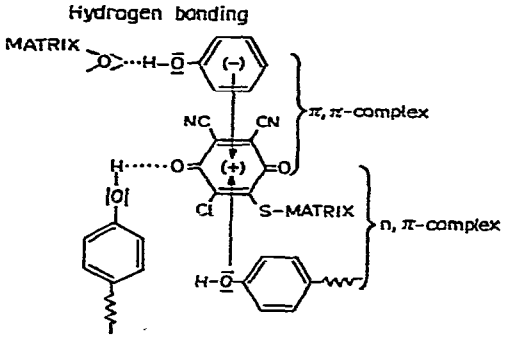
Further insight into the relative importance of charge-transfer forces *vis-à-vis* hydrophobic interaction is provided by the behaviour of the two tripeptides Val–Tyr–Val and Glu–Tyr–Glu. These adsorb equally strongly to pentachlorophenyl-S-Sephadex at 20°, but at 4° Val–Tyr–Val is more weakly adsorbed than is Glu–Tyr–Glu. If the gel was a typically hydrophobic adsorbent the strongly hydrophobic Val-containing peptide should be much more strongly adsorbed than Glu–Tyr–Glu. We may represent the adsorption of a phenol to the pentachlorophenyl-S-Sephadex as follows:



The ANA-Sephadex G-25 gel possibly exhibits weakly ionic properties, as it contains a secondary amino group (although aromatic) and possibly carboxylic groups arising from the hydrolysis of the imide in the alkaline medium used for coupling. The gel seems to behave as a very weak acceptor gel with somewhat stronger hydrophobic properties in comparison with the other adsorbents. It has a low degree of substitution and does not seem to warrant further exploitation.

The dicyanochloroquinone gel is extremely interesting for two reasons. Firstly, the ligand substituent has a very strong electron affinity, and secondly, there is the possibility of hydrogen bonding via the keto groups. Dicyanochloro-*p*-benzoquinone is one of the strongest electron acceptors known. Probably different kinds of n,π-complexes are formed involving, for example, the lone electron pair of the amino group in amino acids and peptides.

The occurrence of ring-substituted hydroxyl groups improves the adsorption considerably, as evidenced by dopamine, serotonin, tyrosine and the tyrosine peptides. The adsorption of tyrosine in acidic solution can be represented as follows:



Chromatography of a synthetic amino acid mixture on DCQ-Sephadex at pH 5.0 (see Fig. 4) reveals some interesting interaction phenomena. A complete separation of the basic amino acids can be obtained. The elution order Lys < His < Arg shows that the degree of basicity of the solutes cannot be the decisive factor responsible for their separation. The π-electrons and/or the lone electron pairs on the nitrogen atoms play an important and probably a dominant role. This again indicates that the adsorption is probably due to charge transfer of π,π- and n,π-types in addition to hydrogen bonding. The carboxylic amino acids Glu and Asp migrate much faster than the neutral non-aromatic amino acids, as is the case on strong polystyrene sulphonate ion exchangers. If the molecules of water of hydration are oriented with their negative oxygen atom towards the free bulk water, there might be an electrostatic repulsion of acidic substances from the bound water layer. This could explain the rapid migration of Glu and Asp, and can also partly explain the retention of the basic amino acids (and the strong retention of tryptamine compared with tryptophan).

The relatively weak adsorption of Tyr and Tyr–Tyr (proved by several experiments, see Table I) shows that if π–π complexation occurs with the phenolate ion it must be rather weak. Furthermore, the quinoid structure might be affected by pH change from acidic to alkaline medium. The alkaline form seems to be a weaker complex former and the ligand might react with strong nucleophiles. The stability conditions should therefore be studied more extensively.

The adsorption of the tripeptides on to DCQ-Sephadex is particularly striking and is also in agreement with the conclusion that electronic charge transfer plays an important role, at least at low pH. The formation of charge-transfer adsorption complexes, possibly with contributions from electrostatic polarization, exchange interactions and dispersion forces, seems to be the main cause for the adsorption of tryptophan, tyrosine and their derivatives. Amino groups and carboxyl groups seem to have a modifying influence on the adsorption, which is primarily governed by the aromatic structure of the solute-adsorbate.

A comparison between ε_{HOMO} and V_E/V_T for some substances tested shows (see Table II) that the correlation is not very impressive, although the trend is clear. The dipole moments for barbituric acid, alloxane, benzimidazole, caffeine and xan-

TABLE II
ELECTRON AFFINITY AND RELATIVE ELUTION VOLUMES ON DCQ-SEPHADEX
200 μmole/g of ligand.

Test substance	ε_{HOMO}	V_E/V_T *(pH 3.2 and 20°)*
Barbituric acid	1.033	1.0
Alloxane	1.033	1.3
Phenylalanine	0.910	1.8
Tyrosine	0.790	2.2
Imidazole	0.660	4.8
Benzimidazole	0.640	7.2
Thiamine	0.596	3.9
Tryptophan	0.530	6.4
Xanthine	0.397	2.1

thine are 1.04, 2.10, 3.90, 4.4 and 4.7 D, respectively. We observe that benzimidazole does not fit into this series either. Nitrogen-containing planar ring systems where the π-electrons are delocalized seem to interact more strongly than expected as judged from the published values for their ε_{HOMO} and dipole moments.

The experiments with the commercial ascorbic acid preparation (Hybrin) as well as chlorpromazine (Figs. 5 and 6) demonstrate how CT chromatography can be used to detect and isolate small amounts of contaminants from highly purified preparations. The presence of the main component in a 100–1000-fold excess does not seem to overshadow the chromatographic patterns to any significant extent.

Heterocyclic compounds which are not well suitable for fractionation by gas or ion-exchange chromatography may be separated on charge-transfer adsorbents, as demonstrated in Figs. 7 and 8. The strong influence of aromatic and pseudo-aromatic π-electron distributions on the adsorption is indicated.

The fact that the chromatographic run is made in an eluent of constant composition and at low temperature eliminates the risk of "chromatographic artifacts". CT chromatography should therefore be very effective in the analysis of physiological fluids such as urine, blood serum, cerebrospinal fluid and plant exudates and also for the analysis of pharmaceuticals, food additives, soft drinks, beer, liquor, natural waters (sea water, surface and drain water) and industrial waste waters. It remains to be seen whether CT chromatography can be further improved for organic and mixed aqueous–organic solvents.

Desalting and "desugaring", as exemplified by the results shown in Fig. 9, demonstrate the possibility of removing efficiently very large amounts of non-adsorbed substances from an aromatic sample substance present in low concentrations. Two fast-moving impurities were removed, but with the volatile buffer at 4° the rear of the main peak indicated the presence of serotonin-like contaminants in fairly large amounts. This result indicates that it is preferable to work at a low temperature and in the presence of a volatile buffer rather than at room temperature and with distilled water as the eluent.

In the literature, reference is often made to strongly time-dependent complex formation requiring several hours or longer[17,31], especially at temperatures below freezing. We have not encountered any complications with respect to the time necessary for adsorption or desorption. In all instances where $V_E/V_T < 10$ we have ob-

served linear chromatography. No systematic attempts have yet been made to minimize the theoretical plate heights of the beds. From the spreading of Trp in Fig. 4 we can estimate the number of theoretical plates to be of the order of 4000–5000. According to Giddings' equation[32]:

$$n = 1 + 0.6N^{1/2}$$

for liquid chromatography, where n is the maximum number of resolvable components and N is the number of plates. On the basis of this equation, we estimate that an 80-cm column of electron-acceptor adsorbent could resolve a mixture of 35 components or more with properly spaced distribution coefficients (association constants). In the V_E/V_T range 0.5–3, a difference of 0.1 V_T units should be sufficient for the complete separation of two solutes on a column of optimal dimensions. With reference to the spreading of the V_E/V_T values shown in Tables I and II, we can therefore take a very optimistic view concerning the future of CT chromatography in aqueous media.

Our programme on charge-transfer adsorption was initiated to meet a demand for improved separation methods applicable to peptides and oligonucleotides. The effects shown for substances with many differently localized donor centres, such as Trp–Trp, Tyr_3 and Phe_3, make CT chromatography very promising. Problems are likely to be encountered in connection with the elution of very strongly adsorbed substances and non-linear chromatographic techniques involving the use of displacers. Such problems are at present under study.

The synthesis of the acceptor gels has not been optimized in any case. It should be possible to increase the yields considerably and perhaps to reach ligand concentrations above 500 μmole/g. Other kinds of synthetic methods might sometimes be preferable, *e.g.*, using strongly reactive ligand substances and an organic reaction medium. It is evident from the results presented here for DCQ-Sephadex, for example, that a higher resolution can be obtained and probably also a much higher capacity. The optimal capacity has not been studied systematically but seems, at least in some instances, to approach the theoretical value calculated from the ligand concentration.

CONCLUSIONS

The adsorption behaviour of electron-acceptor gels is very different from that of ion exchangers and hydrophobic adsorbents. Some preliminary generalizations with regard to the relationships between molecular structure and chromatographic behaviour can be made: (1) aromatic and other planar ring systems promote adsorption; (2) nitrogen with a lone electron pair also promotes adsorption; (3) acidic groups usually tend to weaken adsorption; and (4) several donor centres within the same solute molecule cooperate to give a considerable enhancement in solute–gel interaction.

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